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Dietary protein contains amino acids used in the brain for synthesis of neurotransmitters. Although information on pre- and post-natal exposure to low-protein diets in rodents is available, little is known about effects of such diets on adult animals. Therefore, the behavioral and neurochemical consequences of exposure to a brief (11 days), low (4%) protein diet in animals exposed to normothermic and hypothermic test conditions were examined. In separate groups of animals, the Porsolt Swim test and elevated plus maze were administered. These tasks are sensitive to nutritional and/or environmental manipulations. In other groups of rats exposed to the same dietary and environmental conditions, dopamine, norepinephrine, and serotonin in the striatum were assessed using microdialysis. In the Porsolt swim test, which assesses coping behavior, performance was impaired under normothermic and hyperthermic conditions in animals on the low-protein diet. Performance on the plus maze, a measure of exploration and anxiety, was altered in the hyperthermic condition by low-protein, with the diet increasing exploration. Microdialysis detected increased norepinephrine in the striatum of hyperthermic animals on the low-protein diet. This study demonstrates that changes in stress-related behaviors of adult animals occur following brief exposure to low-protein diets.

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A low-protein diet alters rat behavior and neurotransmission in normothermic and hyperthermic environments

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Abstract

Dietary protein contains amino acids used in the brain for synthesis of neurotransmitters. Although information on pre- and post-natal exposure to low-protein diets in rodents is available, little is known about effects of such diets on adult animals. Therefore, the behavioral and neurochemical consequences of exposure to a brief (11 days), low (4%)-protein diet in animals exposed to normothermic and hyperthermic test conditions were examined. In separate groups of animals, the Porsolt Swim test and elevated plus maze were administered. These tasks are sensitive to nutritional and/or environmental manipulations. In other groups of rats exposed to the same dietary and environmental conditions, dopamine, norepinephrine, epinephrine, and serotonin in the striatum were assessed using microdialysis. In the Porsolt swim test, which assesses coping behavior, performance was impaired under normothermic and hyperthermic conditions in animals on the low-protein diet. Performance on the plus maze, a measure of exploration and anxiety, was altered in the hyperthermic condition by low protein, with the diet increasing exploration. Microdialysis detected increased norepinephrine in the striatium of hyperthermic animals on the low-protein diet. This study demonstrates that changes in stress-related behaviors of adult animals occur following brief exposure to low-protein diets. Published by Elsevier Inc.

Keywords: Anxiety; Amino acids; Tyrosine; Porsolt; Elevated plus maze; Stress

1. Introduction

In rodent models of undernutrition, prenatal and perinatal protein deprivation results in a variety of adverse behavioral, neurochemical and morphological effects [1,2,11]. However, the consequences of protein deprivation on brain metabolism and behavior of adult animals have not been extensively examined. In one of the few studies in this area, rats consuming an 8% protein diet for 8 months displayed morphologic and behavioral deficits, including a loss of neurons in the hippocampus, reduced responsiveness to a novel environment and abnormalities in spatial learning [17]. That study indicated that chronic consumption of a diet deficient in total protein has adverse effects on the central nervous system (CNS), but the effects of short-term consumption of a low-protein diet remain undocumented.

To function adequately, the CNS requires amino acids found in protein foods, including tryptophan, phenylalanine, tyrosine, histidine, glutamine and arginine, as substrates for the synthesis of various neurotransmitters and neuromodulators [4,22]. To date, CNS requirements for protein or specific amino acids have not been systematically investigated because it was assumed that brain requirements for precursor amino acids were not relevant for determining an animal's requirements for dietary protein [13]. However, it has been suggested recently that there may be functional requirements for specific amino acids in the brain and periphery that may be important factors for determination of protein and amino acid requirements [13,22].

Several lines of research suggest that the availability of particular amino acids can be a factor in the regulation of central neurotransmission, cognitive performance and mood

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(for reviews see [13,31]). Furthermore, even brief changes in the availability of several of these amino acid precursors can change brain neurotransmission and behavior. For example, if a single meal deficient in the amino acid tryptophan, the precursor of serotonin (5-hydroxytryptamine; 5-HT), is administered to humans, transient depression-like symptoms are observed [32].

Knowing central nervous system requirements for specific amino acids during periods of undernutrition or when individuals are exposed to highly stressful conditions may be critical. For example, among modestly undernourished, but not highly stressed soldiers participating in a field test of a calorie-deficient ration, decrements in plasma tryptophan were associated with impaired cognitive performance [14]. Furthermore, animal and human studies suggest that tyrosine administration increases brain catecholaminergic neurotransmission and has beneficial effects on behavioral parameters associated with stress resistance [3,6,12,30]. Tyrosine is one of the dietary precursors for brain synthesis of dopamine (DA), norepinephrine (NE) and epinephrine. Wurtman et al. [29] proposed that the neurochemical and behavioral consequences of supplemental tyrosine administration would most readily be observed when humans and other animals are exposed to various environmental and psychological stressors. Subsequent research has confirmed this hypothesis [3,6,15,26,30].

We examined changes in several stress-related behaviors and brain release of neurotransmitters produced by a relatively brief (11 days) exposure to a low (4%)-protein diet in animals exposed to normothermic and stressful (hyperthermic) environmental conditions. The purpose of this study was to determine if relatively brief periods of low protein intake affect selected stress-related behaviors and brain neurotransmitters.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Animal Care and Use Committee. Subjects were male Fischer 344 rats (Harlan Teklad, Madison, WI, USA) weighing ca. 215 g at the start of the experiments. The colony was maintained on a 12L:12D cycle with lights on at 06:00 h. Temperature in the colony rooms was maintained at 24 °C and 30–50% relative humidity. Standard rat chow and water were available ad libitum prior to starting the treatment diets. All testing was conducted between 08:00 and 16:00 h. Different rats were used for each portion of the study ($n = 38$ for Porsolt swim test; $n = 39$ for the elevated plus maze; $n = 33$ for microdialysis) and they were randomly assigned to one of four conditions: (1) low-protein diet, normothermia; (2) low-protein diet, hyperthermia; (3) control diet, normothermia; (4) control diet, hyperthermia. Sample size estimates for each portion of the study were arrived at using appropriate

statistical methods—estimated power was at least 0.8 for each portion of the study.

2.2. Meals

Custom diets were supplied by Harlan Teklad (Madison, WI, USA). The experimental diet was low in protein (4% protein; designated TD93032) and contained 78% carbohydrate and little fat (6%). A control, isocaloric, casein-based diet that contained 20% protein, as well as D,L-methionine for better utilization of other amino acids present (designated TD91352), was also formulated. The control diet consisted of 62% carbohydrate and little fat (5%). Rats were fed either the control diet or test diet for 11 consecutive days ad libitum. Drinking water was freely available. Testing was conducted on the 12th day in normothermic or hyperthermic environmental conditions. Weight and daily food consumption were monitored throughout the experiment, and neither variable differed significantly between test and control diet groups.

2.3. Ambient temperature elevation

All testing was conducted inside an environmental chamber (TESCOR). For the normothermic test conditions, the chamber was maintained at 21 °C with 50% relative humidity. Prior to conducting behavioral testing, rats assigned to a hyperthermia group were placed in a preheated chamber (41 °C) for approximately 1 h. In the microdialysis procedure, rats were brought into a normothermic (21 °C) chamber. After baseline dialysis samples were collected (see below), the chamber was heated to 41 °C. Both rats and chambers reached desired temperatures within 1 h. In both the behavioral and microdialysis procedures, the core body temperature (T_c) was monitored periodically using rectal thermometers. Rats were tested when T_c was elevated by at least 2 °C.

2.4. Porsolt swim test

The Porsolt swim test is a forced swim procedure that assesses unresponsiveness to the environment, failure to cope, or a state of "helplessness" [20]. It is based on the finding that rats, when placed in a tank of water from which they cannot escape, will initially swim vigorously. Eventually, however, the rats will cease escape attempts and float motionlessly while keeping their snouts above water. Immobility induced in this way is selectively attenuated by antidepressant drugs, catecholaminergic agonists and the dietary precursor tyrosine, an effect that does not appear to be attributable to a general increase in locomotor activity [9,20,21,30].

Rats were placed in a cylindrical tank (40 cm height, 25 cm diameter) of water from which there was no escape. Water temperature was maintained at ca. 36.7 °C for both the hyperthermia and normothermia conditions. An individual rat was placed into the tank and observed for a period of 6 min. The total time of immobility in seconds was recorded over the testing session. Two days before testing (i.e., day 10 of the

test or control diet), each rat was observed during the Porsolt swim test under normothermic conditions so that a baseline score could be determined. Mean difference scores (MDS; i.e., test condition minus baseline condition) were computed to determine the total duration of immobility.

2.5. Elevated plus maze test

This test is commonly used to assess anxiety and exploratory behavior in animals exposed to a variety of stressors and drugs that modify anxiety [19]. Rats maintained on low-protein diets, either prenatally or during lactation, exhibit characteristic behavioral changes in the elevated plus maze [1,2]. The elevated plus maze has two open arms and two closed arms. The enclosed arms are surrounded by walls 50 cm in length, and the arms, both open and closed, are 10 cm wide. The entire apparatus is mounted 50 cm above the floor. If the rat chooses to enter a closed arm and remains there for an extended period of time, it is considered an indication of anxiety, whereas entry into an open arm is an indicator of the opposite behavioral state.

All behavioral testing was carried out between the hours of 09:00 and 10:30 in normothermic or hyperthermic conditions. Each rat was placed in the center of the apparatus facing a closed arm. An entry was defined as all four limbs having entered an arm. The number of open-arm and closed-arm entries, as well as time spent in each arm, were observed and recorded by two raters. One rater counted closed-arm entries and time spent in closed-arms while the other recorded the same information for the open-arms.

On heat test days (hyperthermic), the test chamber was heated to 41 °C, 50% humidity, and the rats were given approximately 1 h to reach elevated core body temperature of 40 °C. On no heat test days (normothermic), the test chamber was maintained at 21 °C.

2.6. Microdialysis

Rats were anesthetized with sodium pentobarbital (50 mg/kg/ml, i.p.) and placed in a stereotaxic apparatus. Unilateral guide cannulae (CMA/12) were surgically implanted above the ventral striatum at the following coordinates (A–P: +1.0 mm, L–M: ±3.0 mm, D–V: −4.0 mm from skull) [18]. Guide cannulae were aimed 3.0 mm above the site of injection and were affixed to the skull with anchor screws and dental resin. One week later, microdialysis probes were inserted (D–V: −7.0 mm) into the guides and perfused with artificial cerebrospinal fluid (aCSF) containing the 5-HT uptake inhibitor, citalopram (1 μM; see [8,10,24]). A microinjection pump (CMA/100) was used to deliver the perfusate at 1.5 μl/min. To retard oxidation, cold perchloric acid (PCA; 0.1 N) was added to each collecting vial. Samples (30 ± 5 μl PCA) were collected every 20 min. The

agent, $S(+)$-fenfluramine (FEN; 200 μg over 13 min), was included in the perfusion fluid of all rats according to the procedure of Schwartz et al. [23]. Rats ($T_c \geq 40$ °C) and chambers were heated to test levels within 1 h. Baseline and post-stress measurements were performed while rats were unrestrained in a standard microdialysis apparatus. A total of 297 samples were collected. Samples were immediately placed on dry ice and stored at −80 °C for later analysis. Dialysate samples were analyzed for extracellular DA, NE, epinephrine and 5-HT by HPLC with electrochemical detection [7]. Histological verification of cannula placement into the ventral striatum was performed postmortem.

2.7. Data analysis

Behavioral data on the Porsolt swim test [mean difference score (MDS): test—baseline] and elevated plus maze were analyzed by two-way ANOVA with planned contrast analyses between means. Neurochemical data were analyzed by univariate repeated measures ANOVA with planned contrast analyses between means.

3. Results

3.1. Porsolt swim test

A significant main effect for diet was found for the Porsolt swim test ($F_{1,34} = 15.69$, $p < 0.001$). Contrast analyses revealed that a low-protein diet significantly impaired performance (increased immobility duration) on this test in both hyperthermic and normothermic conditions ($p < 0.001$ and 0.05, respectively; Fig. 1). The low-protein diet impaired performance in both the heat and no heat conditions.

3.2. Elevated plus maze

Behavior in the elevated plus maze was altered by the low-protein diet during hyperthermia. Significant effects on contrast analyses were seen in the amount of time spent in the open arm, $p < 0.01$, as well as the number of entries into the open arms made by the heated rats on the low-protein diet, $p < 0.05$, compared to the normothermic rats receiving the low-protein diet (Fig. 2A and B). The heated, low-protein diet rats spent an increased amount of time in the open arms, and made more entries into the open arms, compared to normothermic animals receiving the same diet.

3.3. Microdialysis

A significant main effect for treatment was found for NE ($F_{3,113} = 2.8$, $p < 0.05$). Contrast analysis revealed that hyper-
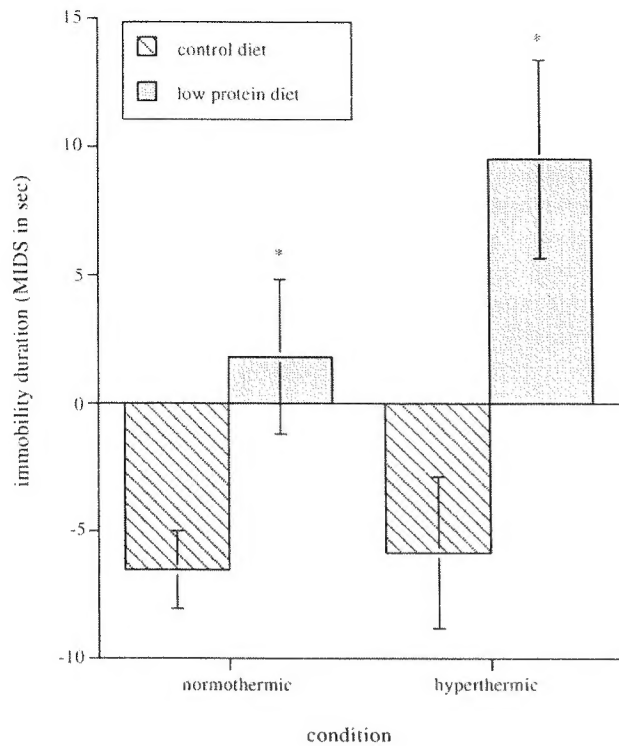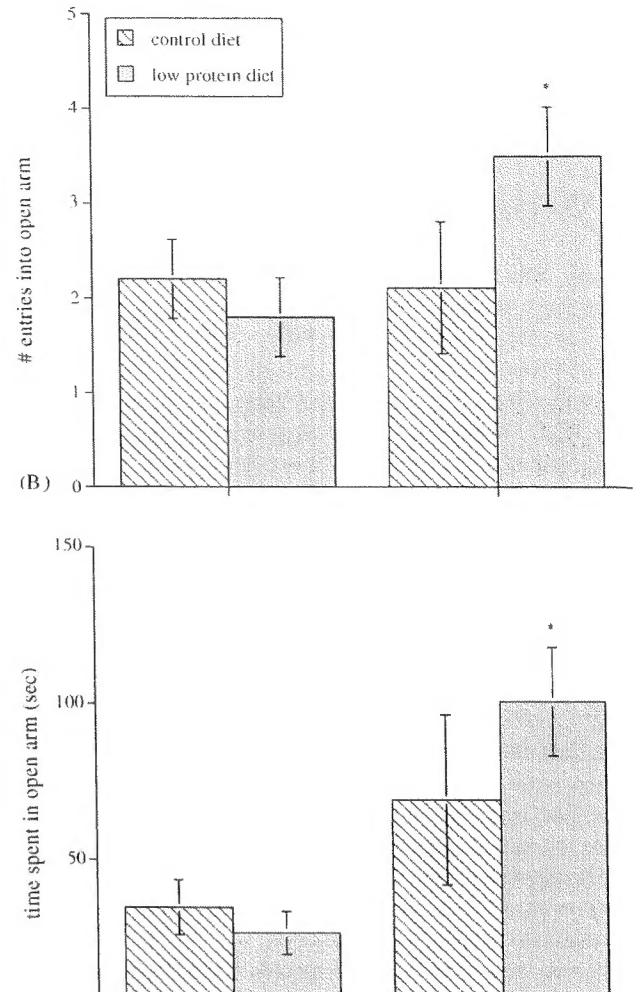
Fig. 1. Data are mean difference scores (MDS) ± S.E.M. for $n = 8$–10 rats/condition on the Porsolt swim test. Significant difference vs. control, no heat and control, heat conditions (*$p < 0.05$ and 0.001, respectively).

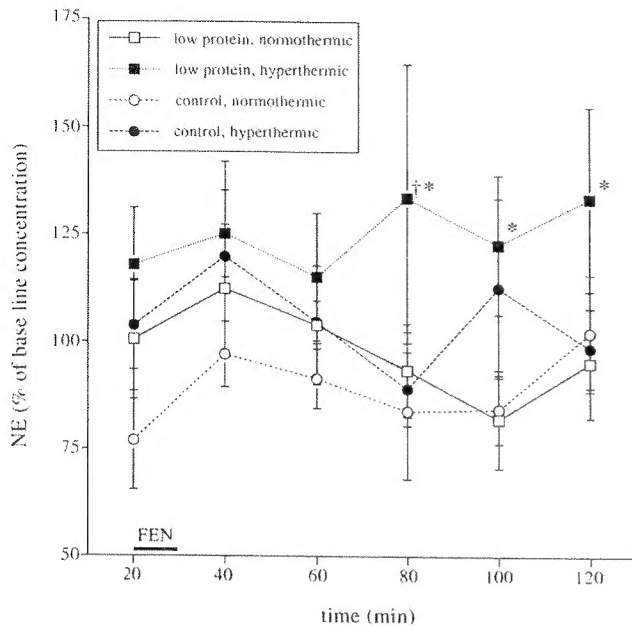rats on the low-protein diet had significantly higher NE concentrations versus heated rats on the control diet at 80 min

Fig. 3. Percentage of mean ± S.E.M. baseline extracellular NE concentration in rat ventral striatum. Significant difference vs. low protein, normothermic rats ($^*p < 0.05$). Significant difference vs. control, hyperthermic rats ($^{\dagger}p < 0.05$).

drugs that decrease anxiety, such as the benzodiazepines [19]. However, food deprivation can increase exploratory behavior even though food restriction can be considered to be a stressor. Various studies have demonstrated that moderate food restriction or removal of essential macronutrients from the diet increase locomotor activity [25,28]. Rats deprived of all protein from their diet for 3 days were continuously active during the dark phase, unlike control rats receiving an 18% protein diet that were only active at irregular intervals [5]. Chronically protein deprived adult rats (consuming an 8% protein diet for 8 months) also exhibit increased exploratory behavior [17]. It is important to note that abnormal behavior in the elevated plus maze was only observed when animals were hyperthermic, and behavioral changes in the Porsolt test were also greater in the heat, suggesting that effects of modest protein deprivation are most readily observed during exposure to stress. This is consistent with studies of the behavioral effects of tyrosine that consistently report these effects only are present in conjunction with exposure to various stressors [3,6,15].

Prenatal and perinatal protein restriction have similar effects to those of acute protein deprivation on behavior in the elevated plus maze, specifically increasing exploration of the open arms of the elevated plus maze [1,2]. The physiological basis for behavioral changes in animals chronically malnourished during brain development and then tested while maintained on an adequate diet are likely to be different than those induced by acute protein deficiency due to permanent brain damage induced by chronic malnutrition [17].

In this study it appears that although levels of stress-related behavior assessed by the Porsolt swim test increase due to a low-protein diet, another behavior normally associated with stress, reduced exploration in the plus maze, increases. This may be due to the fact that increased exploratory behavior as a consequence of food deprivation is an adaptive behavior. Animals engaging in exploratory behaviors are more likely to obtain food. Similarly, an increase in exploration as a consequence of heat stress could be interpreted as an adaptive response, in that increased activity could result in the animals' escape from the adverse environment.

This study demonstrates that a relatively brief period of modest protein deprivation in healthy adult animals can have considerable effects on stress-related behaviors. These effects are amplified by brief exposure to environmental stress and may be related to changes in brain NE transmission. Additional studies to examine other behaviors, neurotransmitter systems and brain gene and protein expression are necessary to clarify the nature of the behavioral changes induced by acute adult protein restriction and the neurochemical and molecular substrates for such changes. In addition, it may be necessary to consider the unique requirements of the central nervous system when dietary requirements for protein and individual amino acids are established.

This study suggests that relatively brief alterations in the protein content of the diet, such as those resulting from various popular weight loss diets, could alter certain aspects of cognitive performance and brain neurotransmission. In addition, consuming ample protein during periods of stress may prevent some of the adverse effects of stress on cognitive performance if the results of the Porsolt test can be generalized to other situations.

Acknowledgments

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